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The roles of redox processes in pea nodule development and senescence

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ABSTRACT

Nodule senescence is triggered by developmental and environmental cues. It is orchestrated through complex but poorly characterized genetic controls that involve changes in the endogenous levels of reactive oxygen species (ROS) and antioxidants. To elucidate the importance of such redox control mechanisms in pea root nodule senescence, redox metabolites were analysed throughout nodule development in a commercial pea variety (*Pisum sativum* cv. Phoenix) inoculated with a commercial rhizobial strain (*Rhizobium leguminosarum* bv. *viciae*). Although a strong positive correlation between nitrogenase activity and nodule ascorbate and glutathione contents was observed, the progressive loss of these metabolites during nodule senescence was not accompanied by an increase in nodule superoxide or hydrogen peroxide. These oxidants were only detected in nodule meristem and cortex tissues, and the abundance of superoxide or hydrogen peroxide strongly declined with age. No evidence could be found of programmed cell death in nodule senescence and the protein carbonyl groups were more or less constant throughout nodule development. Pea nodules appear to have little capacity to synthesize ascorbate *de novo*. L-galactono-1, 4-lactone dehydrogenase (GalLDH), which catalyses the last step of ascorbate synthesis could not be detected in nodules. Moreover, when infiltrated with the substrates L-galactono-1, 4-lactone or L-gulonolactone, ascorbate did not accumulate. These data suggest that ROS, ascorbate and glutathione, which fulfil well recognized, signalling functions in plants, decline in a regulated manner during nodule development. This does not necessarily cause oxidative stress but rather indicates a development-related shift in redox-linked metabolite cross-talk that underpins the development and aging processes.

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INTRODUCTION

Legume root nodules are symbiotic organs housing rhizobial bacteria that fix atmospheric nitrogen. It is now well established that reactive oxygen species (ROS; D'Haeze *et al*. 2003; Matamoros *et al*. 2003; Santos *et al*. 2001; Shaw & Long 2003) and the thiol tripeptide, glutathione (Frendo *et al*. 2005) are part of the repertoire of signals that contribute to the establishment of the legume–rhizobia symbiosis. Many studies have also implicated ROS and antioxidants in nodule senescence (Dalton *et al*. 1991; Dalton, Langeberg & Treneman 1993b; Escuredo *et al*. 1996; Evans *et al*. 1999; Becana *et al*. 2000; Matamoros *et al*. 2003).

Nodules have a much stronger antioxidant defence capacity than the parent roots (Dalton *et al*. 1986; Gogorcena *et al*. 1995; Escuredo *et al*. 1996). As well as abundant ascorbate and reduced glutathione (GSH), nodules have high activities of antioxidant enzymes such as superoxide dismutase, catalases, peroxidases and enzymes of the ascorbate–glutathione cycle [ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR)]. The pioneering studies by Dalton's group (Dalton *et al*. 1986; Dalton, Post & Langeberg 1991) and Evans *et al*. (1999) established that the activities of the ascorbate–glutathione cycle enzymes and the glutathione content were important for nitrogen fixation during soybean nodule development. When soybean roots were supplied with exogenous ascorbate, striking increases in nodule numbers and nitrogenase activities were observed (Swaraj & Garg 1970; Bashor & Dalton 1999). However, of the 800 nodule-induced genes recently reported in *L. japonicus* relatively few (APX, thioredoxin oxidoreductase and peroxiredoxin) are involved in antioxidant metabolism (Colebatch *et al*. 2002, 2004).

In addition to their roles as antioxidants, ascorbate and glutathione have other important functions in plants particularly in the regulation of the cell cycle and growth (Noctor & Foyer 1998; Smirnoff & Wheeler 2000; Arrigoni & de Tullio 2002; Foyer 2004; Conklin & Barth 2004). While the

enzymes of glutathione and homoglutathione synthesis have been characterized in nodules (Dalton *et al*. 1986, 1993a; Matamoros *et al*. 1999b; Frendo *et al*. 2001), there is no data in the literature on whether nodules are able to undertake *de novo* ascorbate synthesis. Three pathways of ascorbate biosynthesis have been proposed in plants, one through L-galactose (Wheeler, Jones & Smirnoff 1998), a second through galacturonic acid (Agius *et al*. 2003), and a third from myo-inositol (Lorence *et al*. 2004). However, the pathway proposed by Wheeler *et al*. (1998) remains the major pathway of ascorbate production in plants, but other routes, even if minor in comparison probably coexist depending on the species and tissue (Davey *et al*. 1999; Agius *et al*. 2003; Wolucka & Van Montagu 2003; Lorence *et al*. 2004; Smirnoff, Running & Gatzek 2004). While an oxidase or dehydrogenase is able to convert L-gulono-1,4-lactone to ascorbate in some plant species, L-galactono-1, 4-lactone dehydrogenase (GalLDH) is the predominant enzyme catalysing the conversion of L-galactono-1, 4-lactone (Gal) to ascorbate in many species (Ôba *et al*. 1995; Østergaard *et al*. 1997; Pallanca & Smirnoff 1999; Siendones *et al*. 1999; Bartoli, Pastori & Foyer 2000). GalLDH is located on the inner mitochondrial membrane, where it uses cytochrome *c* as an electron acceptor (Siendones *et al*. 1999; Bartoli *et al*. 2000). GalLDH is bound to mitochondrial complex I in Arabidopsis and ascorbate synthesis is responsive to respiratory control (Millar *et al*. 2003).

The literature evidence described above shows that ROS accumulation is linked both to the establishment and loss of symbiosis. Moreover, glutathione is essential for root meristem activity such that nodules cannot form in its absence and ascorbate is also required for nodulation and nodule function. To investigate the hypothesis that an imbalance between the levels of nodule oxidants (superoxide and hydrogen peroxide) and antioxidants (particularly ascorbate and glutathione) may lead to oxidative stress and programmed cell death in the developmental ageing process in commercial peas, we examined the developmental time-course of oxidant and antioxidant profiles in the nodules housing commercial rhizobia. We can find no evidence for senescence-induced ROS accumulation, oxidative stress or programmed cell death even though ascorbate and glutathione levels decrease. Rather the data suggest that ROS and antioxidants (ascorbate, glutathione) are important redox components underpinning the plant–rhizobia symbiosis.

MATERIALS AND METHODS

Plant material

Pea seeds (*Pisum sativum* cv. Phöenix) were obtained from Südwestsaat, Germany. Seeds were surface-sterilized and inoculated with *Rhizobium leguminosarum* bv. *viciae* (Becker Underwood, Iowa, USA) according to the manufacturer's instructions. Plants were grown with a 25/19 °C, 70/85% humidity day/night regime, under a 14-h photoperiod with a photosynthetic photon flux density of 600 μmol m^{-2} s^{-1}, on vermiculite in controlled-environment chambers. After 7 d of watering with demineralized water the plants were treated with nitrogen-free Hoagland solution. At the time-points indicated in the text and figures the oldest nodules only were carefully picked from the upper part of the roots, immediately frozen in liquid nitrogen and stored at –80 °C until further use.

Measurement of nitrogenase activity

Nitrogenase activity was assayed by the acetylene reduction assay using excised nodules in sealed vials (Turner & Gibson 1980). We are aware that nitrogenase activity in closed systems can be affected by the assay conditions (Minchin *et al*. 1983), but for comparative purposes and using a short assay time this method provides reliable data.

Measurements of antioxidant enzymes and total soluble protein

Samples were ground in 100 mM Hepes (pH 6.5), 10 mM $MgCl_2$, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ascorbate, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 20% polyvinylpyrrolidone (PVP). GR, MDHAR, DHAR, APX and catalase activities were determined as described by Vanacker, Carver & Foyer (1998). Protein content was estimated with Bio-Rad microassay (Bio-Rad Laboratories, Hemel Hempsted, UK) and bovine serum albumin (BSA) as standard.

Measurement of chlorophyll and antioxidants

Samples were prepared as described in Vanacker *et al*. (1998). Chlorophyll was determined spectrophotometrically via phaeophytin according to Vernon (1960). Ascorbate and DHA were measured according to Foyer, Rowell & Walker (1983). Total glutathione and glutathione disulphide (GSSG) were determined according to Griffiths (1980).

Detection of L-galactono-1,4-lactone dehydrogenase (GalLDH) protein

Proteins were extracted and western blots were performed as described in Bartoli *et al*. (2000) except that nodule samples (0.5 g) were ground in 1 mL of 30 mM 3-[*N*-morpholino]-propanesulfonic acid (MOPS) (pH 7.5), 2 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail. Western blots were probed with specific GalLDH antibody as described in Bartoli *et al*. (2005).

Gal, gulonolactone and ascorbate feeding

Tissues from 3-, 6- and 9-week-old-plants were supplied with a solution containing either 10 mM Na phosphate buffer (pH 7.8) alone or this buffer with either 50 mM Gal, 50 mM gulonolactone or 50 mM ascorbate. Leaves were fed

the solution through the petiole, for 8 h under irradiance (600 μmol /m^{-2} s^{-1}) in the growth cabinet. Nodules and roots were vacuum-infiltrated (30 mbar) with the solutions for 8 h at room temperature. Tissues were then placed in 10 mM Na phosphate buffer (pH 7.8) alone for a further 16 h. Samples were then harvested for ascorbate determinations at the times indicated in the figure legends.

Oxidation state of proteins

The oxidation state of proteins was measured using the Oxyblot kit (Intergen, Edinburgh, UK) according to the supplier's instructions. The ECL detection kit (Amersham Biosciences, Little Chalfont, UK) was used to detect peroxidase activity. The ECL protein molecular weight marker ladder (Amersham Biosciences) was loaded on the same gel, and detected separately, using a streptavidin-horseradish peroxidase conjugate (Amersham Biosciences). Membranes were exposed to film for 12 h (Biomax®, Kodak, Anachem, UK). The non-derivatized controls showed no signal (not shown).

In situ H_2O_2 and O_2 detection

The following reagents were used to detect H_2O_2: 5 mg mL^{-1} 3,3′diaminobenzidine-HCl (DAB; Sigma, Poole, Dorset, UK) in dimethyl sulphoxide (DMSO), diluted 1 : 10 with 10 mM sodium phosphate buffer (pH 7.8); 5 mg mL^{-1} Amplex Red® (Molecular Probes, Leiden, The Netherlands) in DMSO, diluted 1 : 100 with buffer and 0.1% nitrobluetetrazolium (NBT, Sigma) used to detect superoxide. Pea nodules attached to root segments were vacuum-infiltrated with the different stains for 8 h at 30 mbar at room temperature. After infiltration nodules were cut into sections (50 μM). For Amplex Red® treatment sections were washed in 10 mM sodium phosphate buffer (pH 7.8) for 30 min. Sections were observed by conventional light microscopy for DAB and NBT treatments and with a Rhodamine filter for Amplex Red® treatment.

Fixation methods

Nodules were infiltrated under vacuum (40–30 mbar for up to 2 h at RT) in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M Sørensen buffer pH 7.2. After vacuum infiltration fresh fixative was used and the samples fixed over night at 4 °C. At this temperature DNA/RNA are described to be best conserved when formaldehyde is used as a fixative (Srinivasan, Sedmath & Jewell 2002). The samples were washed three times with 0.05 M Sørensen buffer and dehydrated in a series of increasing concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 95, 100% ethanol). After slowly changing the ethanol with Histochoice (Sigma) the samples were embedded in paraffin (Paraplast; Sigma). The wax-embedded samples were left to solidify at 4 °C. Thin sections (10 μm) were cut with a microtome (Reichert-Jung, Vienna, Austria) and placed on TESPA (aminopropyltriethoxysilane, Sigma)-coated slides.

After deparaffinization with Histochoice and rehydration with increasing concentrations of water in a water/ethanol solution, tissue sections were treated either directly with proteinase K (Roche Diagnostics, Lewes, UK, 20 μg mL^{-1} in 10 mM Tris-HCl, pH 7.5, 25 min at 37 °C – time and temperature were optimized) or pretreated with microwave irradiation (1 min at 800 W in citrate phosphate buffer pH 6, then rapidly cooled in water) followed by proteinase K treatment as above (Negoescu *et al*. 1996; Labat-Moleur *et al*. 1998). The slides were rinsed three times for 5 min with PBS (Sigma).

In situ detection of programmed cell death

Nuclei with nicked DNA were determined by terminal deoxynucleotideyl transferase-mediated (TUNEL) labelling, using the apoptosis *in situ* detection kit AP (Roche Diagnostics). The kit allows the detection of nicked DNA by fluorescence microscopy (excitation wavelength about 450–500 nm with detection at about 515–565 nm) after signal conversion. Fast Red (Roche Diagnostics) was used as substrate for signal conversion via alkaline phosphatase. Both results can be compared to avoid false-positive results due to background fluorescence of infected nodule cells. TUNEL labelling was performed according to the manufacturer's instructions. Labelling was terminated by transferring the slides to 0.5 M EDTA. Before the antibody incubations the slides were incubated for 20 min in PBS buffer (pH 7.4 containing 3% BSA and 0.05% Tween 20) to prevent non-specific labelling (Negoescu *et al*. 1996; Labat-Moleur *et al*. 1998; Baima & Sticherling 2002). For the positive control with induced DNA strand breaks prior to the labelling procedures, slides were treated with DNase I grade I (Invitrogen Life Technologies, Renfrew, UK) in 45 μL 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 1 mg mL^{-1} BSA for 20 min at 37 °C. For the negative control, the labelling solution did not contain the terminal transferase. A labelling mixture diluted 1 : 10 with buffer (140 mM cacodylate buffer, pH 7.2, 10 mM Tris HCl, pH 7.2, 1 mM $CoCl_2$) gave an optimal specific signal : background ratio. An Axiovert fluorescence microscope (Zeiss, Oberkochem, Germany) was used in all experiments.

DNA laddering

DNA was extracted from nodules as described in Rouvier *et al*. (1996) with the following modifications. Nodules were ground in 100 mM Tris (pH 8.0) extraction buffer containing 20 mM EDTA, 1.4 M NaCl, 2% cetyl trimethyl ammonium bromide (CTAB), 1% soluble PVP and 2% mercaptoethanol. After a 2-h incubation at 65 °C, samples were centrifuged for 5 min at 7000 *g*. The supernatant was extracted twice in one volume of chloroform:isoamyl alcohol (24 : 1). Samples were then treated with RNAse (20 μg mL^{-1}) for 15 min at 37 °C, and extracted once more. DNA was then precipitated with 1/10th volume of 0.3 M sodium acetate and 0.6 volume of isopropanol for 3 h at – 20 °C. After centrifugation for 1.5 h at 4 °C, 20 000 *g*, the

pellet was first washed with ethanol 70%, dried and then dissolved in TE buffer. Samples (2 μg) of DNA were separated on a 2% agarose gel, stained in ethidium bromide after the run, and visualized under UV light.

RESULTS

The absence of programmed cell death indicators during pea nodule development and senescence

Pea nodules have an indeterminate structure, possessing an active apical meristem that initially yields a cylindrical shape and later a branched structure (Fig. 1a). Five zones have been distinguished in these nodules (as illustrated in Fig. 1b). From the apex these are denoted as Zones I–V. Zone I is made up of small meristematic cells, which are permanently dividing and does not contain bacteria. Zone II houses the infection zone where bacteria are released from the infection threads and differentiate into the bacteroids. In Zone III the bacteria become fully competent in N fixation. Senescence begins in Zone IV, which becomes progressively larger as the nodule develops. In zone V the bacteria do not show the ultra-structural features of bacteroids (Timmers *et al.* 2000). Since internucleosomal DNA fragmentation, indicative of programmed cell death, was observed close to the vascular bundles in 5-week-old soybean nodules (Alesandrini *et al.* 2003), we first explored whether senescence in Zones IV/V of pea nodules showed any evidence of DNA laddering, as a programmed cell death indicator. However, DNA, extracted from nodules, even in the oldest nodules where N-fixation had all but ceased showed no evidence of laddering (data not shown). These data alone do not prove that programmed cell death was absent from pea nodules. We therefore established an *in situ* detection method for programmed cell death localizing nuclei with nicked DNA by terminal deoxynucleotideyl transferase-mediated labelling (TUNEL). While the positive controls showed red staining indicative of DNA fragmentation (Fig. 2a & d) that was evidence from the negative controls (Fig. 2b & d), we found no evidence of TUNEL labelling in any of the tissues of either young (Fig. 2c) or old nodules (Fig. 2f).

Levels of protein oxidation are constant throughout pea nodule development

Since the number of protein carbonyl residues had been shown to increase during the senescence of soybean nod-

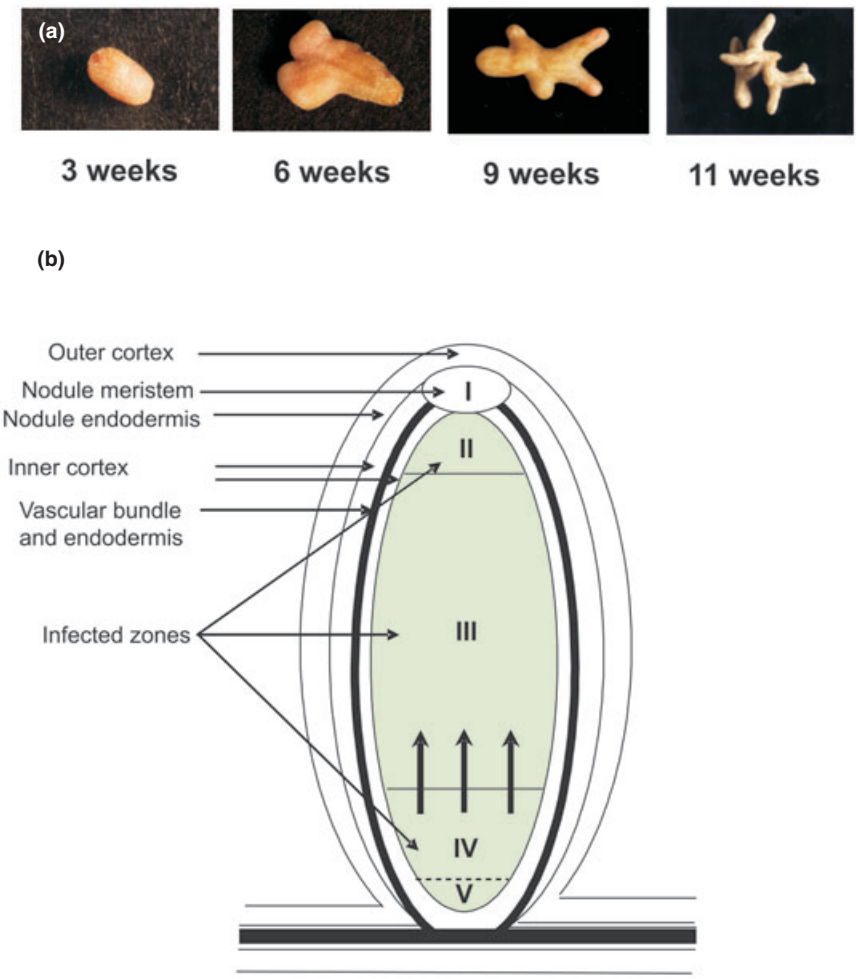

Figure 1. Pea nodule development and structure. (a) The structure of pea nodules harvested from plants at 3, 6, 9 and 11 weeks. (b) A schematic drawing of the internal structure of indeterminate nodules showing the different regions: meristematic zone (I); infection zone (II) nitrogen fixation zone (III); senescent zone (IV); saprophytic zone (V).

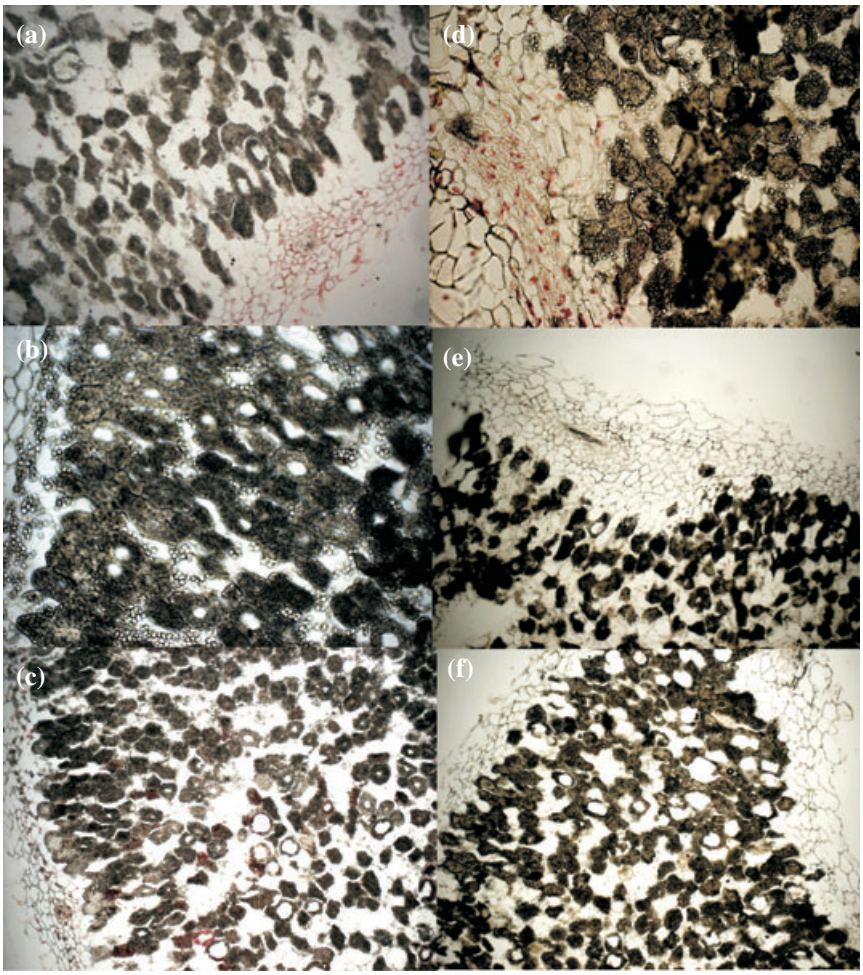

Figure 2. *In situ* detection of programmed cell death. Sections (10 μm) of 5- (a, b, c) and 9- (d, e, f) week-old nodules were stained by TUNEL labelling to detect DNA strand breaks. Positive controls were preincubated with DNAse I (a, d), negative controls treated with labelling solution without terminal transferase (b, e) and untreated 5- (c) and 9- (f) week-old nodules are shown.

ules (Evans *et al*. 1999; Becana & Klucas 1992; Mathieu *et al*. 1998), we determined the oxidation level of nodule proteins via protein carbonyl groups (Fig. 3). However, while protein carbonyl groups were observed at all stages of nodule development there was little change in the overall level or pattern of staining even in the oldest nodules (9–12 weeks; Fig. 3).

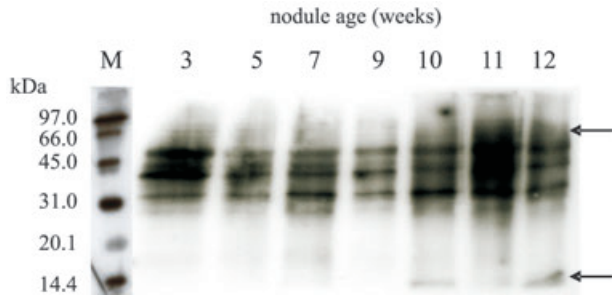

Figure 3. The effect of nodule age on carbonyl group content and distribution on pea nodule proteins. Soluble nodule protein samples (20 μg per lane) from 3- to 12-week-old-nodules were loaded onto each lane together with the ECL protein molecular weight markers (M) for comparison.

Nodule H_2O_2 and O_2^- decrease during nodule development

Since the level of protein carbonyl groups (an indicator of oxidative stress damage to proteins) was relatively constant throughout pea nodule development, we used *in situ* detection methods to determine whether the distribution and levels of oxidants (superoxide and hydrogen peroxide) changed during nodule development. The presence of the blue formazan produced by the reaction of NBT with superoxide, revealed that superoxide was abundant in the apical meristem and the invasion and cortical zones of young nodules but not in other tissue types (Fig. 4a). However, the amount and extent of NBT staining clearly decreased during nodule development (Fig. 4b–d), such that superoxide could only be detected in a small number of cells (Fig. 4d). The decrease or absence of superoxide staining at the last stages of nodule development probably reflect the lack of meristematic activity and the absence of newly invaded cells as the invasion zone region has virtually disappeared in 11-week-old nodules (Fig. 4d).

H_2O_2 was localized using two methods: DAB, which relies on the presence of tissue peroxidases and Amplex

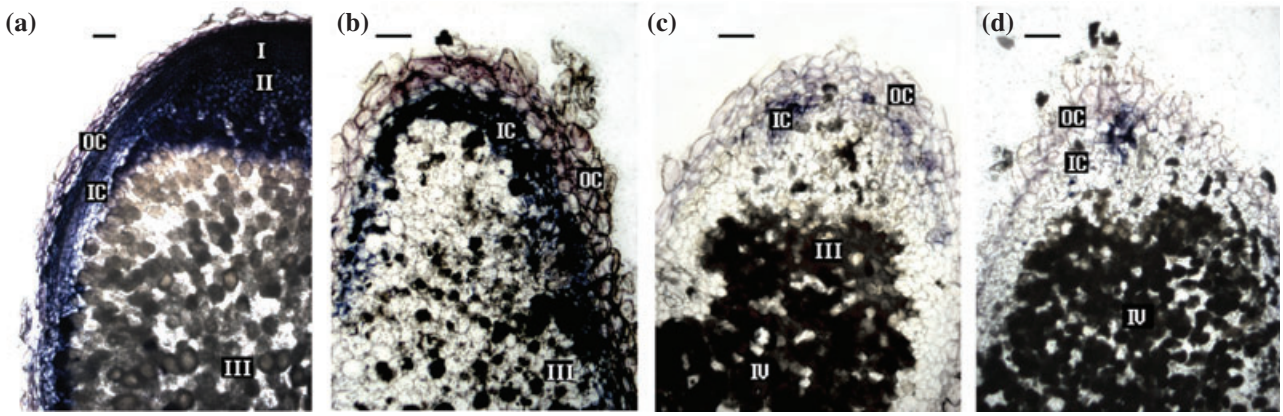

Figure 4. *In situ* localization of superoxide via nitrobluetetrazolium in pea nodules during development. Stained sections from 4-week-old (a), 7-week-old (b), 9-week-old (c) and 11-week-old (d) nodules were observed under the light microscope. The blue coloration indicates superoxide in the meristematic zone, nodule parenchyma (inner cortex – ic) and nodule parenchym (outer cortex – oc). The tissues seen in black are light-dense infected cells.

Red®, which undergoes a rapid chemical reaction with H_2O_2. In young nodules vacuum-infiltrated with DAB, H_2O_2 was detected largely in the meristem and invasion regions (Fig. 5a). In older nodules, H_2O_2 was also detected in the cortex. However, in the 11-week-old nodules H_2O_2, like superoxide, could only be detected in a small number of cells in the cortical tissues (Fig. 5b). Similar results were observed when H_2O_2 was detected with Amplex Red® (Fig. 5c & d). To rule out the possibility that the above stains could not penetrate the endodermis we treated the nodules in the same way with other stains (acridine orange, fuchsin–safranin–astra blue). These stains led to the characteristic coloration in the corresponding tissues throughout the nodule.

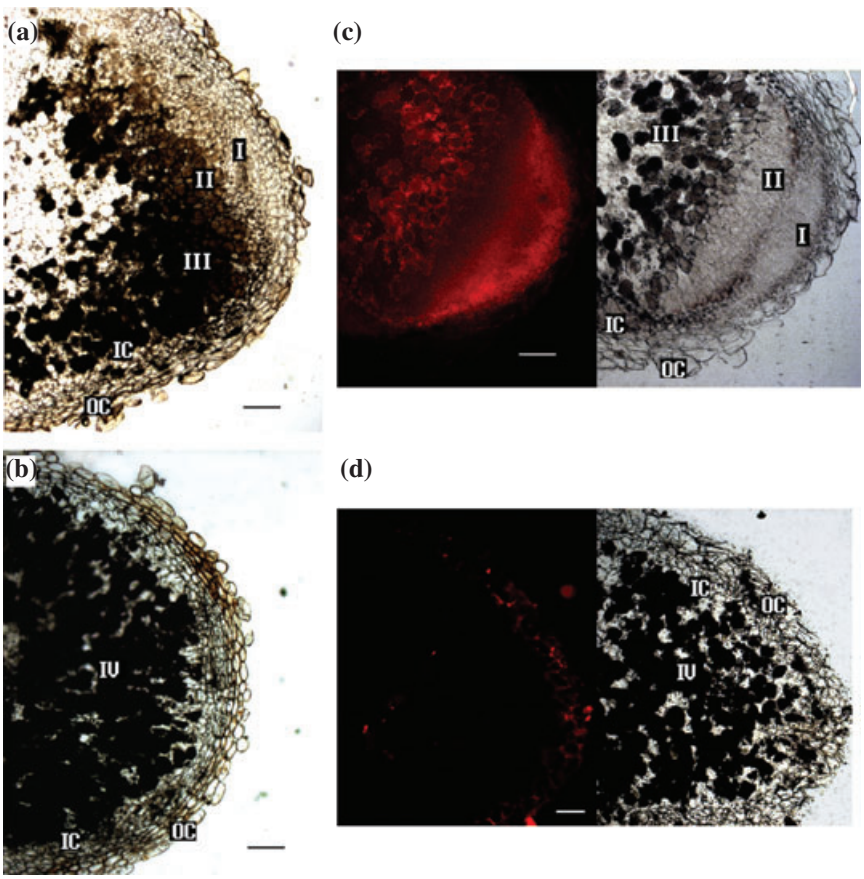

Figure 5. *In situ* detection of H_2O_2 in pea nodules during development. Sections from 5-week-old (a, c) and 11-week-old (b, d) nodules were observed under bright light with a light microscope. The brown colouration indicates H_2O_2 via 3,3′diaminobenzidine-HCl staining (a, b) while the red coloration indicates H_2O_2 with Amplex Red® observed with a rodhamine filter under UV light in the meristematic zone and cortex. Inner cortex (ic), Outer cortex (oc).

Nodule antioxidant enzyme activities

Nodule APX activities were very high at the earliest stage of development and declined to a lower stable value from 5 weeks onwards (Fig. 6a). Root and nodule MDHAR activities were lowest at the earliest stage of development increasing to a maximum at 9 weeks (Fig. 6b). In contrast, DHAR activities were highest at 7 weeks and declined strongly in 12-week-old nodules (Fig. 6c). Nodule GR (Fig. 6d) and catalase (Fig. 6e) activities were relatively constant throughout development, although values were highest between 5 and 9 weeks.

N-fixation capacity shows a strong correlation with nodule ascorbate and glutathione contents

Maximal nitrogenase activity, estimated on either a fresh weight (Fig. 7a) or on a protein basis (data not shown) began to decline from the earliest harvest date (3 weeks) the capacity for nitrogen fixation declining gradually until it fell below detectable levels at 11 weeks (Fig. 7a). The total amount of ascorbate (reduced ascorbate plus dehydroascorbate) also declined progressively over the period of measurement (Fig. 7b). Leaf and root ascorbate also decreased over this time such that the roots of 11-week-old plants had very low amounts of detectable ascorbate (Data not shown). In contrast, root total glutathione (GSH plus glutathione disulphide, GSSG) was constant throughout development (data not shown). The nodule total glutathione pool was greatest at 5 weeks and decreased by about 60% at 9–12 weeks (Fig. 7c). In both roots and nodules the ratios of reduced (ascorbate, GSH) to oxidized (dehydroascorbate, GSSG) antioxidants were fairly constant throughout development (data not shown). In contrast to the activities of antioxidant enzymes which showed little or no correlation with nodule nitrogenase activity (data not shown), there were strong correlations between total nodule ascorbate and nitrogenase activity ($R^2 = 0.68$, Fig. 7b, inset) and also nodule glutathione and nitrogenase activity ($R^2 = 0.75$, Fig. 7a, inset). Unfortunately, we do not have information on the tissue distribution of ascorbate and glutathione content within the nodule. Improved *in situ* staining procedures for these metabolites and/or immunolocalization technologies for glutathione and enzymes involved in antioxidant biosynthesis are required.

Nodules have little ability to synthesize ascorbate synthesis *de novo*

In order to determine whether nodules had the capacity for *de novo* ascorbate synthesis, we firstly probed pea leaf, root and nodule proteins with a polyclonal antiserum that we had raised against *Zea mays* GalLDH. We have previously shown that this antibody recognizes a protein of approximately 64 kDa in mitochondrial membranes from a variety of species (Bartoli *et al*. 2005). The GalLDH antibody detected a single band of approximately 64 kDa in pea leaves and a similar band in roots (but a lower amount),

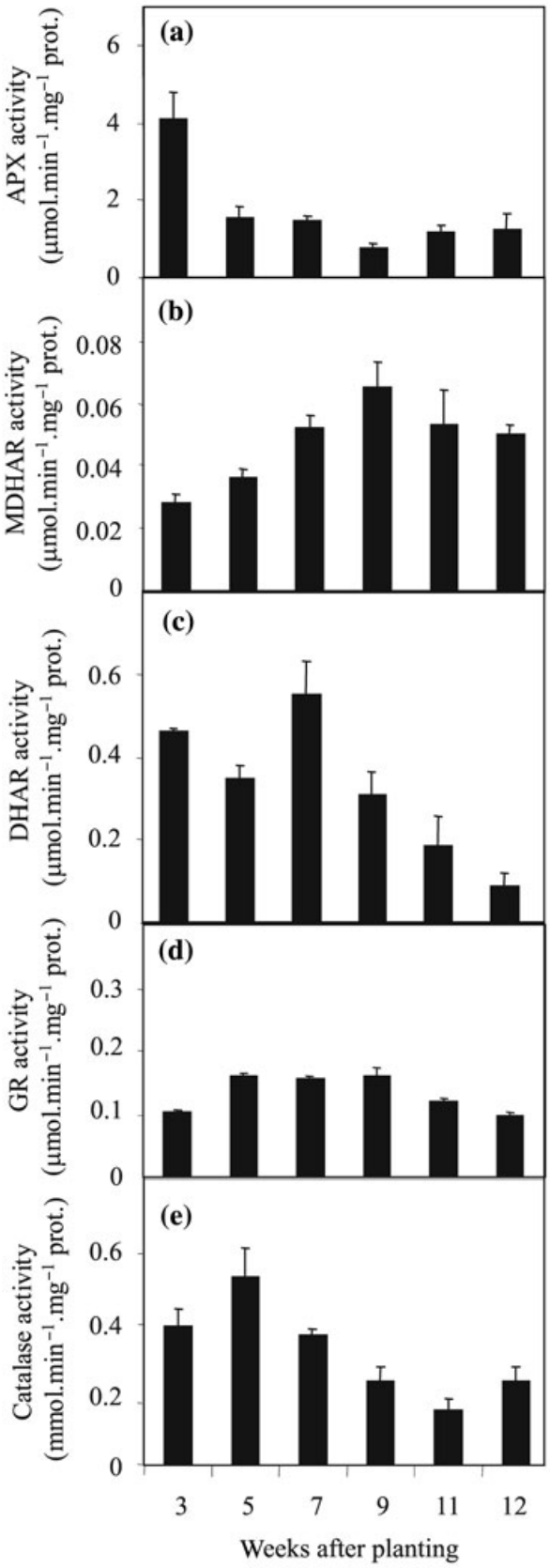

Figure 6. Changes in antioxidant enzyme activities in pea nodules during development. Data are means ± SE from three different plants for each treatment.

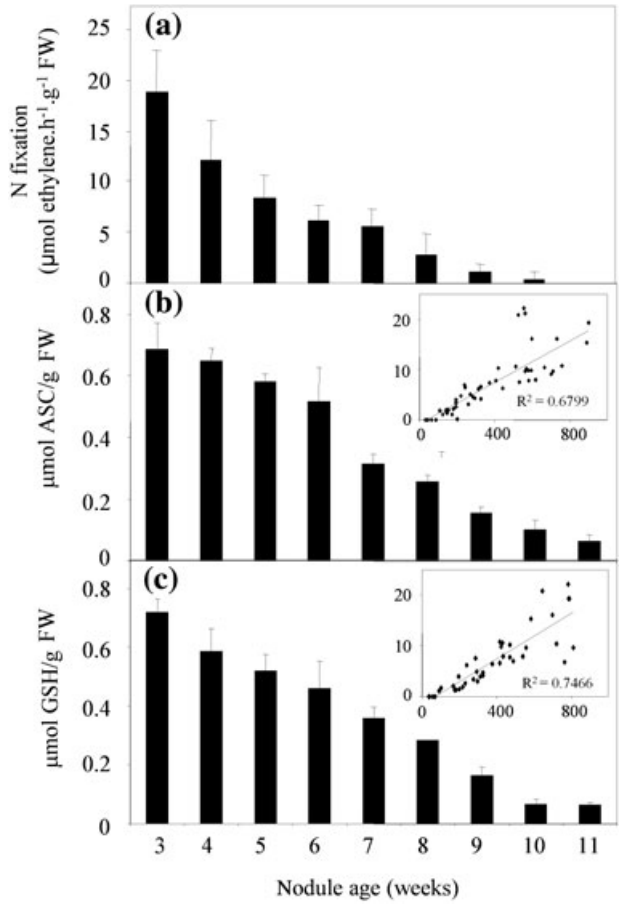

Figure 7. Developmental changes in nitrogenase activity and nodule ascorbate and glutathione contents. Nitrogenase activity (a), measured as acetylene reduction and nodule ascorbate (b) and gluthatione (c) contents were determined throughout the nodule lifespan. Each value in (a) represents the mean (± SE) of 10–15 nodule samples from at least four independent plants, except data at 11 weeks which shows the mean (± SE) of three nodule samples. Each value in (b) and (c) represents the mean ± SE of five samples from at least three independent plants. Insets show the correlation between the decline in nodule nitrogen fixation activity (in μmol ethylene h^{-1} g^{-1} fresh weight: FW) and total nodule ascorbate (b) and total nodule glutathione (c), in μmol g^{-1} FW, during development. The correlation coefficients are given at the bottom of each figure.

but there was no detectable cross-reaction in extracts from pea nodules at any stage of development (data not shown). To explore whether pea nodules can convert the substrate, Gal, into ascorbate, we compared the conversion of substrate into product in pea leaves, roots and nodules at three stages of plant development (3 weeks, Fig. 8a, d & g; 6 weeks, Fig. 8b, e & h; and 10 weeks, Fig. 8c, f & i) and subsequently analysed ascorbate contents after 8 and 24 h incubation. Pea leaves accumulated substantial amounts of ascorbate when supplied with Gal (Fig. 8a–c) in accordance with the known role of leaves as a major source of ascorbate exporting this metabolite throughout the plant (Foyer 2004). The amount of ascorbate accumulated in the leaves of the oldest plants (Fig. 8c) was lower than that of leaves from young plants (Fig. 8a), in agreement with earlier observations that the capacity of leaves to synthesize ascorbate decreases throughout development (Bartoli *et al.* 2000). In contrast, the roots of young pea plants did not accumulate ascorbate when supplied with Gal (Fig. 8d). However, the roots of the oldest plants (Fig. 8f) accumulated ascorbate in the presence of Gal, suggesting that the capacity for ascorbate synthesis is increased in the roots of older plants. It is possible that ascorbate synthesis is induced in roots in response to diminished ascorbate production in leaves and thus roots progressively become a source tissue for ascorbate rather than a sink for this metabolite. In comparison with leaves and roots, nodules had little capacity to accumulate ascorbate when supplied with Gal (Fig. 8g–i). There is, however, a very small increase in level of nodule ascorbate in comparison with the buffer control at the earliest harvest point. This may suggest that very young nodules can synthesize ascorbate from Gal but that this ability is lost as the nodule ages, such that the nodule is a sink for ascorbate imported from the plant from very early in development. This is also suggested by the observation that ascorbate accumulates in nodules when supplied directly in this form (Fig. 8g & i). To explore the possibility of other pathways of ascorbate synthesis we supplied pea tissues with the alternative precursor, L-gulono-1,4-lactone. However, we could find no evidence that pea leaves, roots and nodules could convert L-gulono-1,4-lactone to ascorbate (data not shown).

DISCUSSION

The data presented here show that pea nodule senescence, like leaf senescence (Buchanan-Wollaston 1997) is a highly organized process, orchestrated in an age-dependent manner. Nodule nitrogen-fixation capacity decreased from week 3 of development in parallel with decreases in tissue acorbate and glutathione. However, we could find no evidence to implicate enhanced oxidative stress or programmed cell death in the orchestration of pea nodule senescence. In soybean nodules internucleosomal DNA fragmentation, a marker of programmed cell death, was observed in the central infected zone (Alesandrini *et al.* 2003). Hence, there may be some interspecies variations in the mechanisms underpinning senescence in different types of nodule (Puppo *et al.* 2005).

Superoxide and hydrogen peroxide were detected largely in the nodule cortex and meristematic cells where the initial bacteria/host interactions take place. This is perhaps not surprising given that ROS are important signals exchanged between the bacteria and host cells during nodule initiation (Santos *et al.* 2001; Ramu, Peng & Cook 2002; D'Haeze *et al.* 2003). Whereas hydrogen peroxide and superoxide were detected by *in situ* staining procedures, ascorbate and glutathione levels were determined in whole nodule extracts. It may be that oxidants and antioxidants are spatially separated within the nodule. However, since glutathione is essential for the formation of both the root (Vernoux *et al.* 2000) and nodule (Frendo *et al.* 2005) mer-

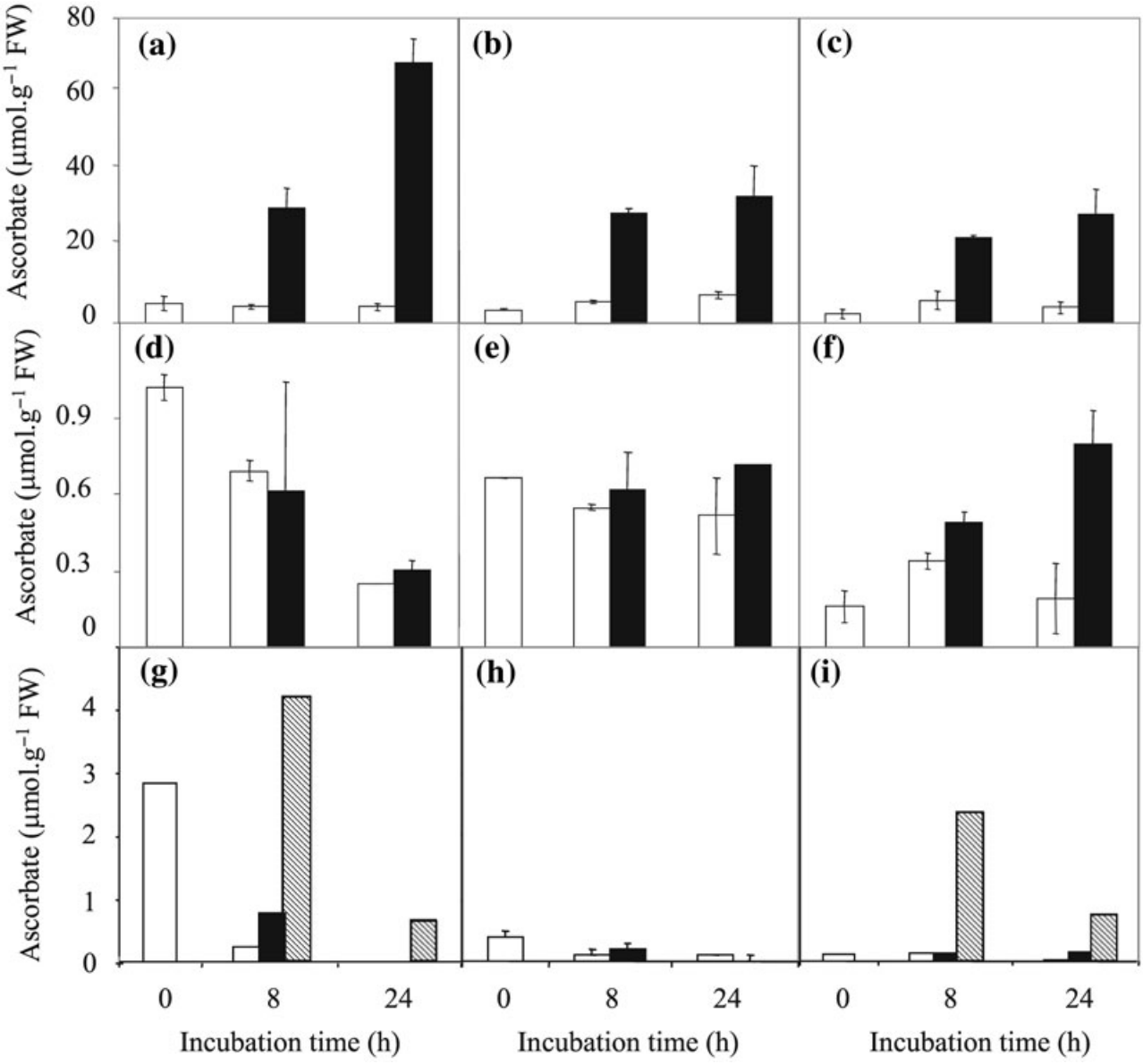

Figure 8. Conversion of Gal to ascorbate in leaves (a, b, c), roots (d, e, f) and nodules (g, h, i). Tissues from 3-week-old (a, d, g), 6-week-old (b, e, h) and 9-week-old (c, f, i) plants were incubated in phosphate buffer alone (white columns) or in phosphate buffer containing 50 mM Gal (black columns). Three-week and 9-week-old nodules were also incubated with 50 mM ascorbate as controls (g, i, hatched columns). Samples were harvested and ascorbate contents measured after 0, 8 and 24 h incubation. Each column represents the mean ± SE of two independent experiments.

istem, it is logical to suggest that glutathione is also abundant in the nodule meristematic cells. Similarly, ascorbate has an important role in the regulation of the plant mitotic cell cycle (Potters *et al*. 2004). Therefore, it is probable that the nodule meristematic cells are also rich in ascorbate. From these observations, we conclude that both oxidants and antioxidants are abundant in the nodule meristem and that a high flux of redox equivalents is characteristic of the pea bacteria/host symbiosis. Since there is no method to date of measuring redox fluxes *in situ*, this can only be confirmed when routine *in situ* staining methods for detecting ascorbate and glutathione in tissues are developed.

It is possible that the observed decreases in the levels of ascorbate and glutathione during nodule development are a consequence (rather than a cause) of the reduction in the number of cell layers forming the meristem and host cell invasion zones. Although there is no evidence to date that superoxide or hydrogen peroxide *per se* modulate the expression of genes involved in ascorbate or glutathione synthesis (Foyer & Noctor 2005), hydrogen peroxide-mediated post-translational regulation of γ-glutamyl cysteine synthetase (the enzyme that catalyses the first step in GSH synthesis) is important in regulating GSH synthesis (Foyer & Noctor 2005). Similarly, ROS can also initiate free radi-

cal-catalysed oxidation of other biomolecules thereby producing a second wave of reactive signalling compounds particularly lipid peroxides, isoprostanes and jasmonic acid. Jasmonic acid is a known regulator of γ-glutamyl cysteine synthetase gene expression and glutathione accumulation. Since ROS and antioxidants decrease more or less in parallel during pea nodule senescence and changes in oxylipin accumulation parallel ROS accumulation, it may be that all these signalling molecules decline in parallel during senescence. Another important signalling molecule, nitric oxide, is decreased in senescent leaves and nodules (Corpas *et al*. 2004). The situation in pea is rather different to soybean, in which hydrogen peroxide, lipid peroxides, protein carbonyl residues and modified DNA base concentrations have all been found in senescent nodule tissues (Evans *et al*. 1999; Becana & Klucas 1992; Mathieu *et al*. 1998). Similar symptoms have been observed in the developmental ageing of lupin nodules (Evans *et al*. 1999; Hernandez-Jimenez, Lucas & Rosario de Felipe 2002) and also in stress-induced nodule senescence (Escuredo *et al*. 1996; Gogorcena *et al*. 1997).

No publications to date have addressed the issue of *de novo* ascorbate synthesis in nodules. Whereas GDP-mannose pyrophosphorylase transcripts were much higher in *L. japonicus* nodules than in the parent roots (Colebatch *et al*. 2002), this enzyme occurs early in the pathway of ascorbate synthesis before it branches from cell wall metabolism. Thus, the high level of GDP-mannose pyrophosphorylase mRNA found in nodules may reflect the structural requirements of nodule formation rather than ascorbate synthesis. Long-distance ascorbate transport through the phloem from the leaves to the roots has been described in *Medicago truncatula* (Franceschi & Tarlyn 2002). Here we have shown that pea nodules do not readily convert Gal or L-gulono-lactone to ascorbate, even though nodules accumulate ascorbate when it is supplied exogenously. Moreover, we were unable to detect GalLDH protein in pea nodules. These results provide the first indication that pea nodules cannot undertake *de novo* ascorbate synthesis, but rather they import ascorbate from the parent plant through the vascular system which is established early in nodule development. It should be noted that the ascorbate content of the parent root declines in parallel with that of the nodule (data not shown) and that it is well known that ascorbate synthesis in leaves is highest in young leaves and lowest in senescent leaves (Bartoli *et al*. 2000; Foyer 2004). Hence, efficient ascorbate transport from the plant and import systems into the nodule tissues are crucial for the maintenance of nodule ascorbate levels.

The observed age-dependent decline in nodule ascorbate and glutathione is similar to that reported in previous studies in pea and other legumes (Dalton *et al*. 1993a, b; Gogorcena *et al*. 1995; Evans *et al*. 1999; Matamoros *et al*. 1999a; Hernandez-Jimenez *et al*. 2002). The thiol contents of nitrate-treated bean nodules rapidly declined but ascorbate contents were relatively constant (Matamoros *et al*. 1999a). However, the total amount of glutathione and the GSH/GSSG ratio was enhanced during natural ageing in lupin nodules (Hernandez-Jimenez *et al*. 2002). The loss of tissue ascorbate and glutathione has previously only been discussed in terms of a decrease in overall antioxidant capacity (Bashor & Dalton 1999; Evans *et al*. 1999; Ross, Kramer & Dalton 1999). However, ascorbate and glutathione have important roles in the modulation of gene expression, senescence and resistance to pathogens (Foyer 2004; Conklin & Barth 2004; Foyer & Noctor 2005). It is probable that these functions, associated with the sensing of redox status and redox buffering capacity, are more important in orchestrating nodule senescence than the antioxidant role of ascorbate. Redox signalling components thus may act in concert with plant hormones to orchestrate the nodule senescence process (Ferguson & Mathesius 2003; Puppo *et al*. 2005).

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